Nucleic Acids Research, 1997, Vol. 25, No. 24 **5125–5126**

Detection of a single base exchange in PCR-amplified DNA fragments using agarose gel electrophoresis containing bisbenzimide–PEG

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Received July 27, 1997; Revised and Accepted October 24, 1997

ABSTRACT

Using PCR fragments of known sequences derived from isolates of two related fungal species, simple submarine electrophoresis in agarose gels containing a bisbenzimide–PEG conjugate (H.A.-Yellow) has been shown to be capable of distinguishing DNA fragments 567 bp long which differ by as little as a single base change. However, only changes affecting bisbenzimide binding sites (which consist of at least four consecutive A/T bases) alter mobility; other changes are ineffective. A second ligand (H.A.-Red) with high G/C specificity is suggested which may be as effective in detecting other sequence changes.

It has been shown that electrophoresis in agarose gels containing bisbenzimide–PEG leads to the separation of PCR-amplified DNA fragments which are identical in length but differ in base composition (1) due to the effects of the PEG bound to the DNA through the bisbenzimide. Sequence similarities ranging between 70 and 90% could be resolved by this method. Müller *et al*. (2) suggested the possibility of detecting point mutations in DNA fragments of 200–300 bp in length but experimental proof that such resolution could be achieved has not been presented. In this paper we show that the method can indeed be used for the detection of minute changes in base composition and even for the detection of point mutations.

PCR-amplified DNA fragments from five different isolates of the plant pathogenic fungi *Verticillium alboatrum* and *Verticillium dahliae* (3) have been studied and products differing by as little as a single base change resolved. Standard primers (3,4) were used to amplify fragments of 567 bp containing the ITS1 and ITS2 regions, the intervening 5.8S ribosomal RNA (rRNA) gene and small sections of the adjacent large and small subunit rRNA genes. Differences in base composition between DNA fragments varied from 1 to 7 nt (0.18–1.23%) and in AT content by 0–0.88%. Figure 1 illustrates how fragments which could not be separated by normal electrophoresis (Fig. 1A) could be resolved by bisbenzimide–PEG–agarose gel electrophoresis (Fig. 1B). Table 1 lists the differences between the sequences of the PCR fragments. In the presence of bisbenzimide–PEG, the DNA fragments from *V.alboatrum* isolates 1974, 235 and 220 all migrate slower than the fragments from *V.dahliae* 1928 and 1871 and are clearly separated from them in the electrophoresis. The first three differ from the second two by 4–7 nt (0.71–1.23%). The DNA fragments from

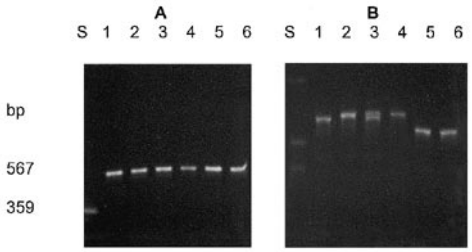

Figure 1. (**A**) PCR fragments (567 bp long) corresponding to the ITS1/5.8S rRNA/ITS2 region (and short adjacent sequences from the flanking large and small sub-unit rRNA genes) from: lane 1, *V.alboatrum* 1974; lane 2, *V.alboatrum* 235; lane 3, an equimolar mixture of fragments from *V.alboatrum* 1974 and 235; lane 4, *V.alboatrum* 220; lane 5, *V.dahliae* 1928; lane 6, *V.dahliae* 1871 run in an agarose gel containing 1.5% (w/v) agarose (H.A. Agarose Molecular Biology Grade, Hanse Analytik GmbH, Bremen, Germany) in 0.5× TBE pH 7.5, with U = 5.7 V/cm for 3 h. Lane S, DNA size standard containing three DNA fragments each 359 bp long but different in base composition (H.A. DNA Standard I, Hanse Analytik). Neither the fungal fragments nor those in the markers are resolved because separation is according to length. (**B**) PCR fragments and size standard as in (A) but electrophoresed in an agarose gel containing bisbenzimide–PEG (0.45 µM H.A.-Yellow, Hanse Analytik). Buffer and conditions were otherwise identical. Clear separations of the marker fragments and of the fungal fragments based on differences in base composition as described in the text are shown.

V.alboatrum isolates 1974 and 235 differ in only one position, a proportional change in sequence similarity and in AT content of 0.18%; these two fragments have been resolved. The DNA fragments from *V.alboatrum* isolates 1974 and 220 are also separated; these fragments differ by 2 nt (0.35%) but do not differ in AT content as the exchanges compensate each other. Two further point mutations could not be detected viz. single nucleotide exchanges between the PCR fragments from *V.alboatrum* 235 and 220 and between those from *V.dahliae* 1928 and 1871.

Binding sites for bisbenzimide, a minor groove binding ligand, contain at least four consecutive AT residues (5,6) but the binding affinity varies widely between the different arrangements of AT base pairs (7). Our results can be interpreted in accordance with these studies which suggest why some nucleotide exchanges will affect the separation in this method while others will be ineffective. Clearly, a nucleotide exchange will have an effect only if a binding site of bisbenzimide is generated or lost by that exchange. For example, the single base difference between the PCR fragments from *V.alboatrum* isolates 1974 and 235 (TTcAT→TTtAT) generates a new binding site for bisbenzimide (shown underlined)

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within the fragment from isolate 235 giving a relative decrease in electrophoretic mobility of this fragment, due the increased amount of PEG bound, and thereby allowing the separation of these PCR fragments. Conversely, the single nucleotide exchange in the fragments from *V.alboatrum* 235 and 220 (CaC→CgC) does not result in different mobilities because no binding site for bisbenzimide is affected. Despite the apparent contradiction that they do not differ in overall AT content, *V.alboatrum* isolates 1974 and 220, which differ at two positions, can be separated electrophoretically; one exchange affects mobility (TTcAT→TTtAT) whilst the other does not (CaC→CgC). The fragment from *V.alboatrum* 1974 shows larger than expected differences in electrophoretic mobility relative to the fragments from *V.dahliae* 1928 and 1871. This may be because the effective change in the sequence TAaTTA→TAgTTA causes the loss of an AATT site, which has the highest affinity to bisbenzimide of all sites (7) and this may give a greater separation of the PCR fragments than a single effective change might suggest. The fragments from *V.alboatrum* 220 and 235 differ from those of *V.dahliae* 1928 and 1871 by two effective base exchanges (and three or four ineffective exchanges) and the best separation seen in these experiments is between these pairs.

These results demonstrate that small changes in base composition, and even single nucleotide exchanges, within DNA fragments of 567 bp can be detected. However, the separation of any particular pair of fragments depends not primarily on the number of nucleotide exchanges nor on the change in AT content but mainly on the context of the exchange(s) in a given sequence.

In order to separate DNA fragments that differ in base exchanges that do not affect the binding of bisbenzimide it may be possible to use other DNA binding ligands; 4-carboxy-malachite green coupled to PEG (H.A.-Green, Hanse Analytik) is another AT-binding ligand (2) whilst 10-phenyl neutral red is a DNA binding ligand with a high GC specificity (8). Coupled to PEG, this ligand (H.A.-Red, Hanse Analytik) can be used for separating DNA fragments according to changes in GC sequence motifs (2) but the effectiveness of this ligand for detecting small sequence changes has still to be confirmed empirically.

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Table 1. Nucleotide exchanges between the PCR fragments derived from isolates of *V.alboatrum* and *V.dahliae*

	V.alboatrum 235	*V.alboatrum* 220	*V.dahliae* 1928	*V.dahliae* 1871
V.alboatrum 1974	CTTTATTcATAC→CTTTATTtATAC	CTTTATTcATAC→CTTTATTtATAC	GTAaTTAC→GTAgTTAC[a]	GTAaTTAC→GTAgTTAC[a]
		CaC→CgC	CTtTaTTcATAC→CtcTgTTtATAC	CTtTaTTcATAC→CTcTgTTtATAC
			CcTTG→CaTTG	GTaC→GTcC
			GTaC→GTcC	CaC→CgC
			CaC→CgC	
	235 has one more effective A/T;	220 has one more effective A/T;	1974 has one more effective A/T;	1974 has one more effective A/T;
	1974 runs ahead	1974 runs ahead	1974 runs behind	1974 runs behind
V.alboatrum 235		CaC→CgC	CTtTa→CTcTG[b]	CTtTa→CTcTg[b]
			GTAaTTAC→GTAgTTAC[a]	GTAaTTAC→GTAgTTAC[a]
			GTaC→GTcC	GTaC→GTcC
			CaC→CgC	CaC→CgC
			CcTTG→CaTTG	
		No effective A/T changes;	235 has two more effective A/T;	235 has two more effective A/T;
		no separation	235 runs behind	235 runs behind
V.alboatrum 220			CTtTa→CTcTg[b]	CTtTa→CTcTg[b]
			GTAaTTAC→9GTAgTTAC[a]	GTAaTTAC→GTAgTTAC[a]
			GTaC→GTcC	GTaC→GTcC
			CcTTG→CaTTG	
			220 has two more effective A/T;	220 has two more effective A/T;
			220 runs behind	220 runs behind
V.dahliae 1928				CaTTG→CcTTG
				No effective A/T changes;
				no separation

Base exchanges are indicated by lower case letters. Binding sites for bisbenzimide are underlined.

[a]The loss of the very high affinity binding site (AATT) from *V.alboatrum* 1974 may cause a greater relative decrease in electrophoretic mobility than a single effective change might suggest, allowing better separation between the PCR fragments from 1928 and 1871. This effect can be observed in all comparisons involving *V.alboatrum* 220, 235 and 1974 with other isolates (but not between themselves).

[b]In the comparisons involving *V.dahliae* 1871 and 1928 with other isolates (but not between themselves) there are two changes separated by a single base, both individually apparently causing the loss of a binding site in the two *V.dahliae* isolates relative to *V.alboatrum*. However, as these sites would overlap it is assumed that the pair of changes act in concert causing the loss of only one site (CTtTa→CTcTg) for a net change of two sites not three as a simple count might suggest.